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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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TITLE OF THE INVENTION (500 characters max)

A method for the production of intervertebral disc derived chondrocyte transplants....

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[Page 1 of 2]

Respectfully submitted,

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Date February 12, 2004

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A method for the production of intervertebral disc derived chondrocyte transplants and the use as a transplantation material

Abstract

The invention relates to a method for the in vitro production of vital intervertebral disc chondrocyte or cartilage transplants, and the transplantation of the in vitro produced intervertebral disc chondrocytes and cartilage. According to this invention, viable chondrocytes, obtained from human and/or animal degenerated or damaged intervertebral disc, are cultured in cell culture vessels until a sufficient amount of cells with original native phenotype and high proliferation and differentiation capability are available that were then transplanted into a degenerated or damaged disc.

Description

The invention relates to a method for I) the in vitro production of vital intervertebral disc derived chondrocyte transplants, made of chondrocytes obtained from degenerated or damaged intervertebral disc tissue of patients suffering on spine disorders, and to do use thereof as a transplantation material for the treatment of degenerated or damaged intervertebral disc and for III) the in vitro

production of three-dimensional, vital and mechanically stable intervertebral disc cartilage tissue and their use as a transplantation material for the treatment of degenerated or damaged intervertebral disc, and for the IV) method of transplantation, V) as well as for their use in testing/screening of active substances and physical factors.

The invention relates also to in vitro produced intervertebral disc derived chondrocyte transplants and in vitro produced three-dimensional intervertebral disc cartilage as well as therapeutic formulations, such as injectabilia which contain the produced tissue and cell transplants.

The progressive intervertebral disc degeneration, caused by trauma or normal aging process, leads to acute and chronic back and leg pain as well as to instability of spine. Long-term physical disability and a reduced quality of life are results of progressive degeneration of disc. More than 300.000 patients in Europe suffering on spine dissorders. Because no effective therapies to retard or reverse disc degeneration have yet been devised, a variety of surgical procedures have been developed to treat disc degeneration and back pain. Unfortunatelly, the procedures currently available fail to offer an outcome that is prosthetic and at the same time physiologic. They include surgical removing of nucleus compartiment with intact annulus of discus hernia from the spinal chanel or the removing of complete intervertebral disc following by insertion of implants or following by fusion of both adjacent spinal segments. However, the surgeries tends to limit motion by

immobilisation of affected spine level and their outcome is a lack of function at particular spine segment followed by excessive stresses to adjacent intervertebral discs.

The invention describe for the first time the unique method for the production intervertebral disc derived chondrocyte transplants, that allows, by the transplantation of cells into the degenerated or damaged intervertebral disc, the regeneration of the intervertebral disc and efficient maintenance of the disc matrix and therefore restore neurological and biomechanical function disorder caused by discus hernia of affected spine segment.
Furthermore, the invention describe for the first time the method that allows the regeneration of disc and maintenance of neurological, biological and mechanical function of disc even in progressive intervertebral disc degeneration or traumatical damage of outer ring of disc (annulus fibrosus),
With the former method described is directed to the production of intervetrebral disc derived chondrocyte transplants, whereas the latter method described is focused on the production of three-dimensional intervetrebral disc tissue transplant, however both methods use autologous (patient own) cells isolated from damaged intervertebral disc.

The autologous chondrocyte transplantation is the well established method for the treatment of focal articular cartilage defects by using cultured autologous articular cartilage cell transplants. The method is based on

capability of articular chondrocyte transplants for the de novo formation of articular cartilage in vivo.

In this method, for example, a biopsy is taken from a patient from a region of hyaline healthy cartilage, chondrocytes are isolated from the biopsy, propagated by culturing and finally transplantated into articular focal cartilage defect using injection. The treated cartilage lesion site is completely filled-up with de novo synthesed articular cartilage produced by transplanted chondrocytes.

It is well known, that cultured autologous cell transplants are capable to regenerate the specific target tissue in vivo following their in vivo application into damaged site in the body.

The goal of Tissue Engineering in the regeneration of intervertebral disc is the restoration of degenerated or damaged disc tissue by the use of ethical and medical innovative method: transplantation of an specific cell transplant or transplantation of ex-vivo manufactured three-dimensional disc cartilage. There is no published similar methods.

The task of this invention was therefore to provide such a methods for the production of intervertebral disc derived chondrocyte transplants and methods for the production of vital and mechanically stable intervertebral disc tissue suitable for the transplantation and rapid disc regeneration and maintenance of disc function. For the finding/creating the described method, it was essential that the biopsy obtaining can be performed under ethical conditions and that the cultured disc derived cells maintain their unchanged phenotype as well as have a high proliferation rate and differentiation capacity.

According to the described invention, the source for the manufacturing of transplant is degenerated and/or damaged intervertebral disc tissue, because no other tissue source, for example such as adjacent intact adult intervertebral disc, is available for the described treatment method, due to ethical and medical reason. Due to apoptosis of tissue-specific cells and their replacement by unspecific, various type of cells in degenerated or damaged tissue, so far it was suspected, that it is not possible to obtain sufficient number of vital cells from the degenerated or damaged tissue, which are capable to proliferate with sufficient multiplication factor and finally able to differentiate to the specific tissue and to restore intervertebral disc. Surprisingly, it has been found, that the sufficient number of vital cells can be isolated from degenerated or damaged intervertebral disc tissue and that those isolated cells also under given culturing conditions proliferate and differentiate tissue-specific and therefore are suitable for cell-based therapy for the functional restoration of damaged disc.

For the treatment of spine by the manufactured disc derived chondrocyte transplants it is important that the outer disc ring, annulus fibrosus, is allready healed in that way that no liquid, such as cell suspension, can come out from the internal compartiment of the disc. That time period is patient-specific. During this time period the cultured autologous disc-derived chondrocyte transplants may not change their tissue-specific phenotype regarding their

differentiation capability and by that the treatment success.
Furthermore, the in vitro manufactured cell- and tissue transplants should not express immunological reactions in the patient who is receiving the transplant.

Surprisingly, we found that this requirement can be fulfilled by using of simple method as described in claim 1.

According to the invention, patient-derived tissue biopsies or samples, e.g. from damaged and degenerated intervertebral discs are used as starting material. The disc-building cells are isolated from this biopsies, according to conventional methods, using enzymatic digestion of the tissue, migration, or reagents recognizing the target cells. According to the invention, these cells are than subjected to culturing in suspension in a simple fashion, using conventional culture medium supplemented by patient serum, without supplementing antibiotics and fungistatics as well as growth factors, in a cell culture vessels until sufficient cell number become available. The period of culturing and the number of cell passaging were reduced as much as possible in order to maintain the unchanged cell phenotype. Such phenotype changes have been observed in long-term cell cultures with frequent passages (see Fig 4). After sufficient cell number has been achieved, the cell were harvested and the cell transplant as the suspension of disc-derived chondrocytes has been produced.

According to the invention, in an additional method, the isolated disc-derived chondrocytes have been precultured and without passaging propagated in short-term. Following this, the precultured cells have been harvested and cryopreserved until the transplantation. Before the transplantation, the cell have been thawed and cultured in the conventional cell culturing medium in the presence of autologous serum until sufficient number has been achieved. Following this, the cells have been harvested and the autologous disc-derived chondrocyte transplant has been produced. Surprisingly, it has been found, that the disc-derived cells did not lost through freezing, thawing and finally short-term culturing, their capability to synthesize specific matrix compounds (see Fig. 5). However, the disc-derived cells lose their capability to produce de novo matrix compounds in long-term monolayer culturing up to 2-3 month without freezing.

According to the invention, in an third method, the disc biopsies have been obtained from damaged or degenerated discs of patients. The tissue-building (forming) cells are isolated from the biopsies, according to conventional methods, using enzymatic digestion of the tissue, migration, or reagents recognizing the target cells. According to the invention, these cells are then subjected to stationary culturing in monolayer simple fashion, using conventional culture medium, until an sufficient number is achieved. The cultured cells are then transferred in cell culture vessels with hydrophobic surface and tapering bottom and subjected to the culturing until a three-dimensional cell aggregate is formed which includes at least 40% by volume, preferably at least 60% by volume and

up to a maximum of 95% by volume of extracellular matrix (ECM) having differentiated cells embedded therein. The cell aggregate having formed has an outer region wherein cells capable of proliferation and migration are present. The structure of the cell aggregates obtained according to the invention is illustrated my the microscopic photographs in Fig 1. Fig.1 shows a detail enlargement of the cross-section of a according to the invention produced cell aggregate with M as the zone of reduced cell proliferation and the tissue-specific matrix proteins and the zone P as the outer zone of proliferative and migratory cells.

It is noteworthy that all the cells derived from damaged and degenerated disc tissue maintain their high proliferation capacity (Fig 2.) as well as extremely high differentiation potential for the synthesis of disc-specific matrix proteins and marker proteins, such as Aggrecan (Fig. 3a), hyalin-specific proteoglycans (Fig. 3b), collagen type I (Fig. 3c), collagen type II (Fig. 3d) collagen type III (Fig. 3e) und protein S100 (Fig. 3f) and do not lose their phenotype by freezing and subsequently thawing (Fig. 5).

It is surprisingly, that all disc-derived cells, according to this invention, isolated from damaged and degenerated disc tissue and integrated in the three-dimensional disc-cell aggregates produced from those isolated cells according to the invention survive, and that the cells integrated inside the aggregates do not necrosize even after an advanced period of culturing. With increasing time of cultivation, the cells inside the aggregates undergo

differentiation to form aggregates of disc cartilage tissue consisting of ECM, differentiated disc-chondrocytes and peripheral proliferating disc-chondrocytes. During the cell differentiation in cell culture, the spacing of the aggregated cells increases due to formation of the tissue specific matrix. A tissue histology develops inside the in vitro produced three-dimensional disc aggregates is highly similar to natural tissue. During the further course of disc aggregate production, a zone of cells capable of proliferation and migration is formed at the boundary of the disc aggregates. This zone is invaluably advantageous in that following incorporation of the produced disc aggregates into the damaged intervertebral disc, the cells located in this peripheral zone are capable of migrating to make active contact with the surrounding tissue and/or enable integration of the tissue produced in vitro in the environment thereof. Thus, the produced disc-tissue-specific cell aggregates are excellently suitable for use in the treatment of damaged and degenerated intervertebral disc and in the in vivo neogenesis of intervertebral disc.

Due to biomechanical loading of intervertebral discs immediately following the treatment by disc cell transplants and also according to the primary goal of the treatment to restore the disc height by transplanting of the disc cell transplants, it could be advantageous to use larger, mechanically stable in vitro produced three-dimensional tissue for the treatment. For this case, at least two or preferably more of the cell aggregates obtained are fused by prolonging culturing thereof under

the same conditions and in the same culture vessels as described above until the desired size is reached.

Fig. 4 shows five disc-cell aggregates during their fusion. The boundary between single disc cell aggregates cannot be recognized any longer. Following further culturing, the disc cell aggregates are completely fused and a larger disc tissue patch has formed. The structure of the larger disc cell aggregates thus obtained is identical to that of the single disc cell aggregates obtained initially. They may include ECM up to a maximum of 95%, and all of the cells included in the piece of tissue obtained exhibit vitality.

A medium usual both for suspension and monolayer culture, e.g. Dulbecco's MEM supplemented with serum, can be used as a cell culture medium. It is preferred to use DMEM and HAMS at a ratio of 1:1. However, to avoid an immunological response of the patient to the tissue produced in vitro, it is preferred to use autogenous/autologous serum from the patient as serum. It is also possible to use xenogeneic or allogeneic serum.

According to the invention, no antibiotic, fungistatic agents or other auxiliary substances are added to the cell culture medium. It has been found that only autogenous, xenogenic or allogenic cultivation of the cells and cell aggregates and cultivation in absence of antibiotic and fungiststaic agents allow for non-affected morphology and differentiation of the cells in the monolayer culture and undisturbed formation of specific matrix within the cell

aggregates. Furthermore, by avoiding any additive during the production, any immunological reaction is excluded following incorporating the in vitro produced tissue in a human or animal organism.

The size of in vitro produced disc cartilage depends on the number of introduced cells per volume of culture medium. For example, at incorporating $1x10^7$ cells in 300 µl culture medium, three-dimensional disc cartilage aggregates with approximately 500-700 µm diameter, are formed within one week. Another way would be in vitro fusion of small cell aggregates to form larger ones - as described above - and incorporation of the latter in the defect. According to the invention, it is preferred to use between 1×10^4 and 1×10^7 cells in 300 µl culture medium to produce the small cell aggregates, more preferably 1×10^5 cells. Depending on the cell type and patient-specific characteristics, the cell aggregates having formed after several days are then subjected to the culturing for at least 2-4 weeks to induce formation of the tissue specific matrix. From about one week of culturing on, it is possible to fuse individual disc cartilage tissue in special cases, so as to increase the size of tissue patch.

As cell culture vessels, the inventive cultivation in suspension requires the use of those having a hydrophobic, i.e., adhesion-preventing surface, such as polystyrene or Teflon. Cell culture vessels with a non-hydrophobic surface can be made hydrophobic by coating with agar or agarose. Further additives are not required. Preferably, well plates are used as cell culture vessels. For example, 96-well

plates can be used to produce small cell aggregates, and 24-well plates to produce fused larger aggregates.

The invention is also directed to therapeutic formulations comprising the intervertebral disc chondrocyte transplants and intervertebral disc cartilage transplants according to the invention, e.g. injection solutions.

The invention is also directed to the use of intervertebral disc chondrocyte transplants and intervertebral disc cartilage transplants of the invention for the testing/screening various factors, e.g. active substances and physical factors having an effect (influencing) on the formation of matrix and differentiation of cells. For this purpose, the intervertebral disc chondrocyte aggregates are produced according to the invention, the potential drugs, therapeutic substances and compounds and medicinal products to be tested are added at various stage of maturity, and most various parameters of in vitro intervertebral disc tissue formation and maturation are characterized. Compared to conventional drug testing using animals or tumour systems, these tests are highly patients-specific and enable individual results and diagnosis as a result of using autologous material only.

Without intending to be limiting, the invention will be illustrated in more detail below with reference to the examples.

Examples

Example 1: In vitro production of intervertebral disc chondrocyte transplants

A biopsy is taken from damaged or degenerated intervertebral disc tissue. Disc-derived chondrocytes are isolated from this biopsy using enzymatic digestion by incubation with collagenase solution. Following separation of the isolated cells from the undigested tissue, the cells are transferred in cell culture flasks and, following addition of DMEM/HAMS F12 culture medium (1:1) and 10% autologous serum from the patient, incubated at 37°C and 5% CO_2. The medium is exchanged twice a week. After reaching the confluence stage, the cell layer is washed with physiological saline solution and harvested from the cell culture surface using trypsin. Following subsequently washing, the disc-derived chondrocytes are transferred into physiological saline solution and the disc chondrocyte transplant is so available for the transplantation.

The differentiation capacity of disc-derived chondrocytes in the released disc chondrocyte transplant has been shown in an in vitro model. Due to the disc-specific matrix proteins and marker proteins, expressed in the cells of the released disc chondrocyte transplants, the disc-specific tissue structure is formed (Fig.3)

Example 2: Transplantation of intervertebral disc chondrocytes

The produced intervertebral disc chondrocyte transplants, as described in example 1, containing minimally 1.000, max. 100 Million cells, preferably approx. 1 Million

intervertebral disc chondrocytes, were taken up in physiological saline solution and injected into a interspace of damaged and/or degenerated intervertebral disc. It has been shown/determined that in the treated intervertebral disc, the water content increases and the disc height can be maintained, due to the transplanted intervertebral disc chondrocytes producing de novo matrix proteins. According to the invention, the in vitro produced disc chondrocyte transplants are well accepted from the from the treated patient. The in vitro produced disc chondrocyte transplants ensures rapid integration of the proliferating and migrating disc cells as well as the regeneration of disc tissue through their differentiation capability.

Therefore, the disc chondrocyte transplants allow with their features the rapid regeneration of the intervertebral disc, the rapid healing/recovery of the patient and the rapid functional restoration of the intervertebral disc.

Example 3: in vitro production of intervertrebral disc tissue

A biopsy is taken from damaged or degenerated intervertebral disc tissue. Disc-derived chondrocytes are isolated from this biopsy using enzymatic digestion by incubation with collagenase solution. Following separation of the isolated cells from the undigested tissue, the cells are transferred in cell culture flasks and, following addition of DMEM/HAMS F12 culture medium (1:1) and 10% autologous serum from the patient, incubated at 37°C and 5% CO_2. The medium is exchanged twice a week. After reaching the confluence stage, the cell layer is washed with

physiological saline solution and harvested from the cell culture surface using trypsin. Following subsequently washing, 1 x 10^5 disc-derived chondrocytes each time are transferred into cell culturing vessel coated by agarose. After one day, the first cells arrange into aggregates. Those aggregates are supplied with fresh medium every second day and cultured for at least 2 weeks.

In those, according to the invention, in vitro produced intervertebral disc tissue, the expression and deposition of disc-specific matrix compounds and marker proteins such as Aggrecan (Fig. 3a), hyalin-specific proteoglykanes (Fig. 3b), Collagen Typ I (Fig. 3c), Collagen Typ II (Fig. 3d), Collagen Typ III (Fig. 3e) und Protein S100 (Fig. 3f) is shown. Those molecules are components of the native disc tissue in vivo, representing the most important structural proteins which are of crucial significance for disc function.

Example 4: Transplantation of produced disc tissue

The disc tissue produced as described in example 3 (approx. 10 to 1000 patches), preferably up to 100 patches were taken up in physiological saline solution and injected into the intervertebral space of progressively degenerated and/or strong traumatically damaged intervertebral disc with destroyed annulus fibrosus. It has been determined, that, in according to the invention, in vitro produced intervertebral disc tissue is well accepted from the treated patient and that the transplanted tissue allows, beside the mechanical restoration of the affected disc tissue, also rapid integration into that tissue due to

proliferating and migrating cells in outer periphery of aggregates, as well that transplanted tissue allows the regeneration of affected tissue due to differentiation capacity of the cells in the transplant. Therefore, the structure and the function of the in vitro produced disc tissue allow rapid regeneration of the affected disc tissue, the rapid healing and recovery of the patient and the rapid restoration of the disc function.

Claims

1. A method for the production of disc chondrocyte transplants, characterized in that disc chondrocytes are isolated/obtained from a damaged, degenerated and sequestered intervertebral disc tissue, and these cells are grown under maintaining their phenotypical characteristics, that those cells can differentiate and therefore are able to regenerate the intervertebral disc tissue following their transplantation .

2. The method according to claim 1, Characterized in that the intervertebral disc chondrocytes can be isolated from a damaged, degenerated and sequestered intervertebral disc tissue

3. The method according to claim 1, Characterized in that the isolated intervertebral disc chondrocytes are grown under strictly auotologous culture conditions, e.g. only supplementing autogeneous, patient-own, serum

4. The method according to claim 1, characterized in that the isolated intervertebral disc chondrocytes are grown in that way, that their phenotypical features regarding their capacity to synthese the matrix proteins and marker proteins remain unchanged.

5. The method according to claim 1, characterized in that, the isolated intervertebral disc chondrocytes after initial short-term culturing can be frozen and subsequently thowed, without changing their phenotypical features regarding their capacity for synthesis of matrix-specific proteins and marker proteins.

6. The method according to claim 1 Characterized in that, the isolated intervertebral disc chondrocytes, grown in a monolayer culture, express their capability for the formation of extracellular matrix comprising of disc-matrix-specific proteins.

7. The method according to claim 1, characterized in that, the isolated intervertebral disc chondrocytes, initially grown in a monolayer culture, and subsequently frozen and thowed, maintain their capacity for the formation of extracellular matrix comprising of disc-matrix-specific proteins.

8. The use of intervertebral disc chondrocyte transplants according to claim 1 as autogenous, xenogeneic or allogeneic transplantation material for the treatment of degenerated intervertebral disc.

9. A therapeutic formulation, comprising intervertebral disc chondrocyte transplants according to claim 1.

10. The method according to claim 1, characterized in that, the intervertebral disc chondrocyte and in vitro produced intervertebral disc tissue also can be cultured with addition of growth-stimulating compounds.

Abstract

The invention relates to a method for the in vitro production of interbertebral disc chondrocyte transplants, produced from disc chondrocytes isolated from damaged, degenerated and sequestered intervertebral disc, where the cells can be grown without changes of their specific features, and the cells maintain their proliferation and differentiation capacity regarding the formation of intervertebral disc-specific matrix and the cells can be transplanted.
The invention is also directed to the in vitro produced intervertebral disc tissue and therapeutic formulations, e.g. injection solutions comprising such tissue.

Surgical procedure for the autologous disc-derived chondrocyte transplantation (ADCT)

This surgical intervention is the application of manufactured disc-derived chondrocyte transplants – transplantation- into the damaged disc. It has to be performed after healing and achieving of stability of the ligamentum longitudinale posterior.

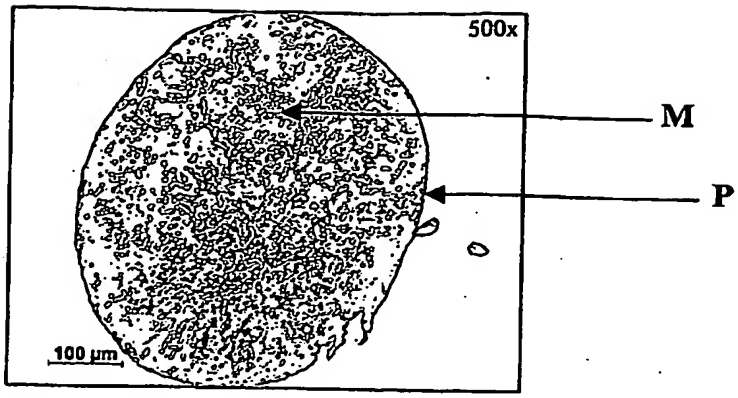
Surgical procedure has to be performed in surgical sterile tract marking of the intervertebral disc space on the patient back under fluoroscopy control into 2 dimensions,

following skin desinfection, covering the skin areal with a sterile foil under use of superficial local anesthesia with Xylocain 1% or comparable drug.

It follows determination of the syringe volume for rinsing after the cell instillation, puncture of the opposite side of the former surgical approach, insertion of the Initial-Puncture-Needle-Threesquare with lancet,, removing of the lancet, positioning of the penetration needle, manipulations under fluoroscopy control, performance of the pressure measurement according to the pressure measurement working guideline, resuspension of the cryo-vial with the cell suspension, instillation of the cells without using contrast media and without (not recommended but possible) radiological control to avoid adverse effects on the cells, injection of 0,1 ml NaCl-solution to rinse remained cells out of the syringe, removing of the application system and local wound care.

Post operative care

Includes up to 3 days hospitalization with up to 12 hours strict bed rest, following 12 to 24 hours regular bed rest, following 24 to 48 hours bed rest with isometric exercises (physiotherapy, site positioning, abdomen positioning possible). From 2nd to 21st day a stable lumbar orthesis is prescribed.



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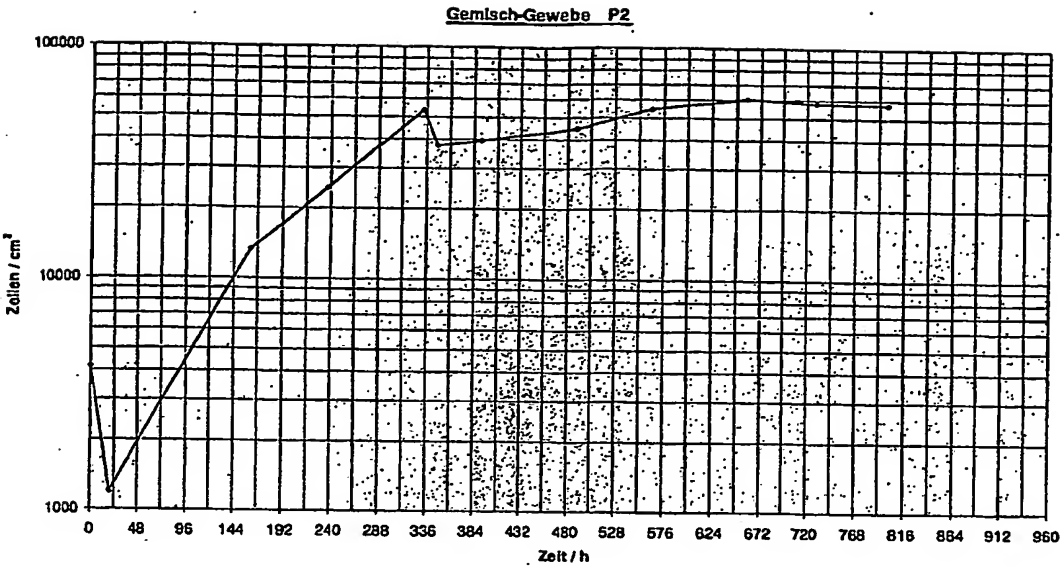

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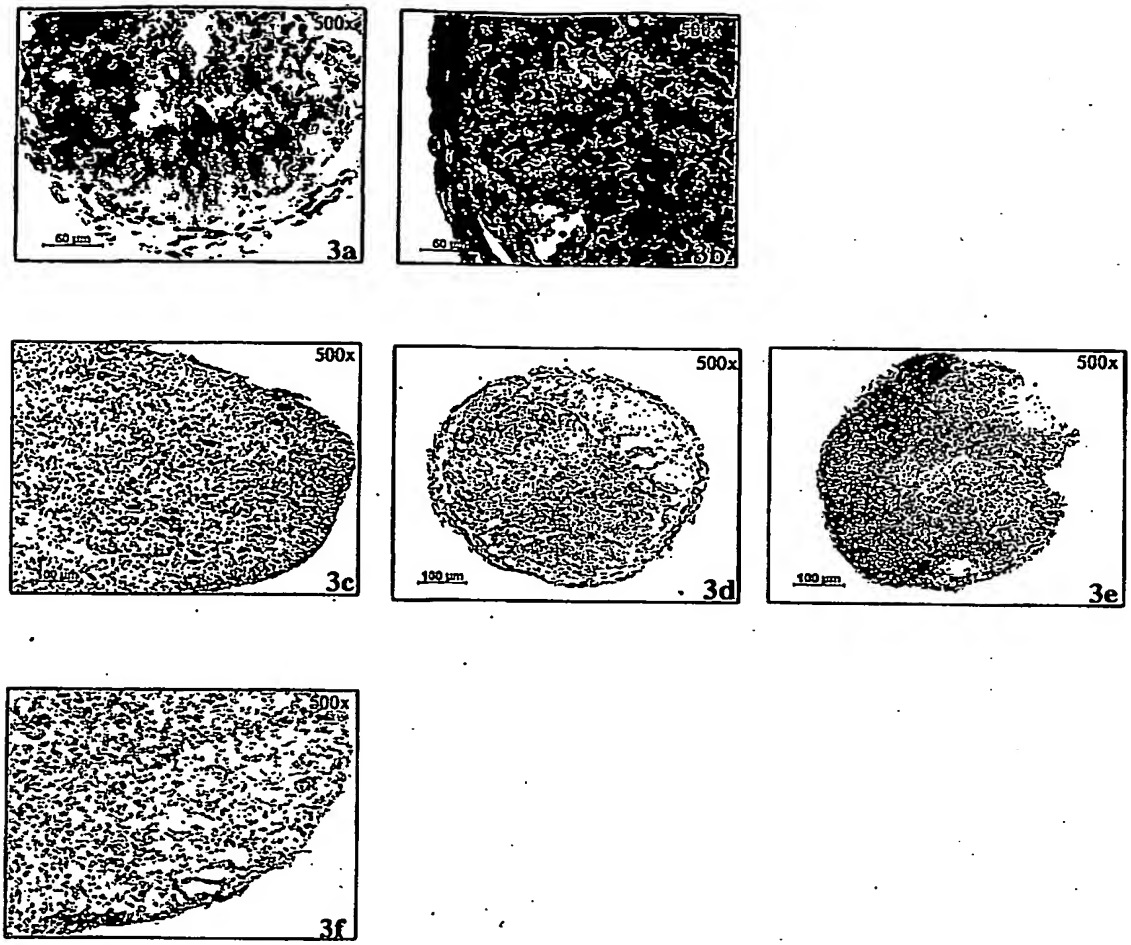

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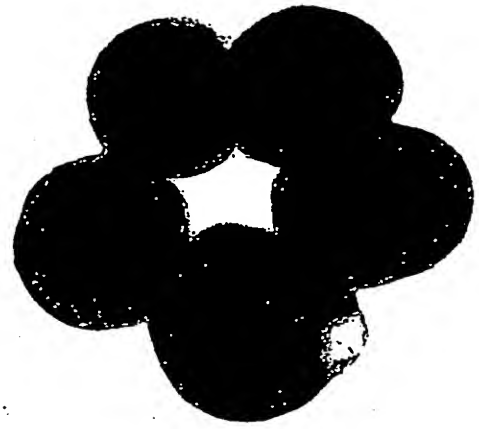

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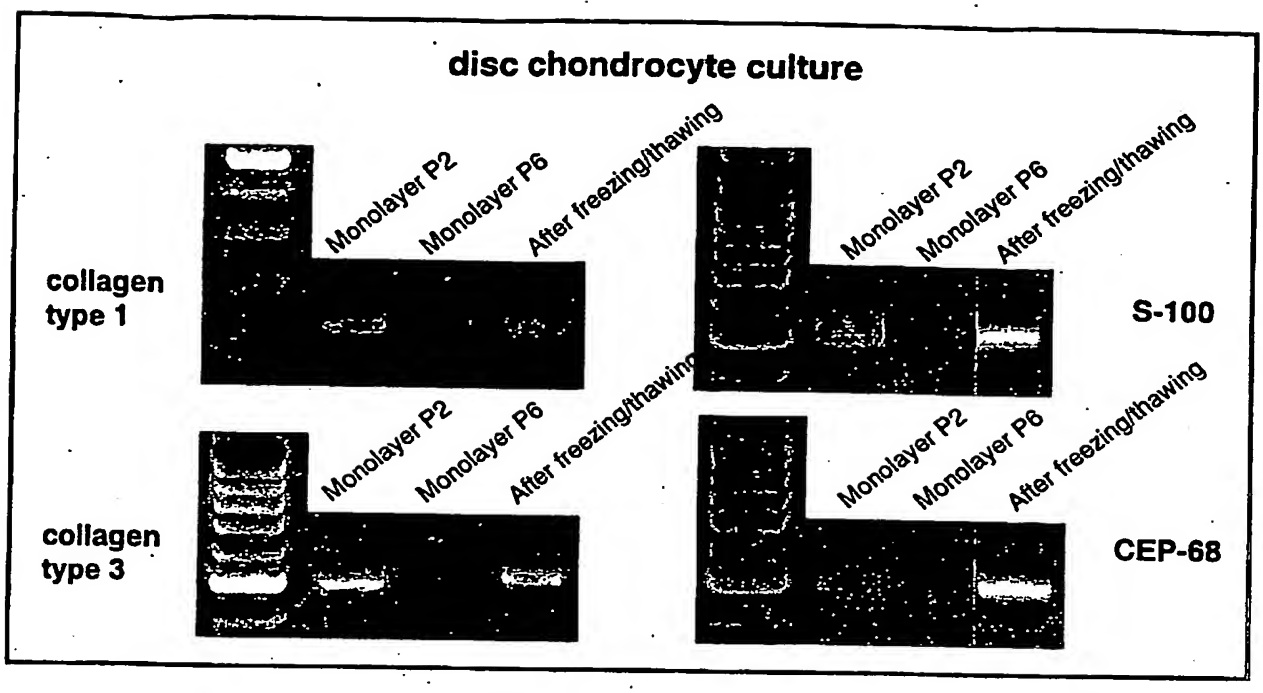

Fig. 5: Expression of different matrix and regulative proteins by disc derived chondrocytes cultured in monolayer for different passages and cultured in monolayer after freezing and thawing of cells. Monolayer passage 2 (P2), Monolayer passage 6 (P6), after freezing and thawing (after freezing/thawing).